J. Clin. Chem. Clin. Biochem.
Vol. 28, 1990, pp. 459–463

Aluminium Binding to Serum Constituents: A Role for Transferrin and for Citrate

By *M. F. van Ginkel*[1], *G. B. van der Voet*[1], *H. G. van Eijk*[2] and *F. A. de Wolff*[1]

[1] *Laboratorium voor Toxicologie, Academisch Ziekenhuis Leiden, Leiden, Nederland*
[2] *Chemische Pathologie, Erasmus Universiteit, Rotterdam, Nederland*

(Received October 23, 1989/April 12, 1990)

Summary: The binding of aluminium in rat serum was studied. Rats were loaded intraperitoneally with different doses of aluminium(III)chloride 4 times during one week, before being killed by cardiac puncture.

One ml of serum was applied to a Sephacryl S-200 SF column and 70 fractions were collected. In the collected fractions, the distribution of aluminium was measured and compared with the concentrations of total protein, transferrin, and citrate.

The presence of a high molecular weight aluminium-complex in serum is confirmed. Although a possible role for albumin cannot be excluded, it is most likely that transferrin plays a role as a carrier for biological transport of aluminium in plasma. In addition to transferrin, aluminium was shown to be associated with citrate in serum, resulting in a low-molecular weight complex. It is postulated that citrate acts as a chelator for aluminium, and that the Al/citrate complex in serum may play an important role in intracellular accumulation, and hence the toxicity, of aluminium.

Introduction

It is generally accepted that aluminium is the cause of the dialysis-related diseases, microcytic anaemia, vitamin D-resistant osteomalacia, and dialysis encephalopathy (1–3). Controversial, however, is the role of aluminium in the pathogenesis of some neurological disorders such as *Alzheimer*'s disease, amyotrophic lateral sclerosis, and *Parkinson*ian dementia (4–7).

Mammalian toxicity of aluminium is dependent on the bioavailability as well as on the chemical form of the element. Although the chemical speciation of aluminium in serum may be essential for understanding the (neuro-)toxic effects of this metal, little interest has been given to this aspect. Speciation studies using gel filtration chromatography, have indicated an association of aluminium with albumin (8, 9) and transferrin (10–12). *King* and co-workers (8, 9), and *Bertholf* and co-workers (13) also reported the association of aluminium with low-molecular weight serum constituents. Furthermore, the ultrafiltrable low-molecular weight fraction is reported to increase with the total serum aluminium concentration (9, 14). The existence of a low-molecular weight aluminium species, however, has been challenged by others (10–12).

The aim of this investigation was, therefore, to examine the speciation of aluminium in rat serum in relation to the total serum aluminium concentration, using a study for calcium speciation as a methodological model (15).

Materials and Methods

Animals

Female Wistar rats weighing between 200–230 g were fed a standard diet (Hope Farms, Linschoten, The Netherlands) with tap water ad libitum (aluminium concentration less than 10 µg/l).

The animals were given 4 intraperitoneal injections of 1 ml each, consisting of physiological saline containing different concentrations of aluminium ($AlCl_3 \times 6H_2O$), on day 1, 3, 4, and 7. The dosage scheme was thus chosen not to study a dose-response relationship, but to achieve a range of serum concentrations. Therefore, two rats received a total dose of 0.8 mg aluminium (1 mg per kg body weight every 48 h), another 2 rats 4 mg aluminium (5 mg/kg body weight every 48 h), and 2 rats 8 mg aluminium (10 mg/kg body weight weight every 48 h). A control group of 4 rats received saline only. One rat receiving 5 mg/kg body weight died, so that the total number of animals for further investigation amounted to 9.

On day 8, the animals were killed by cardiac puncture under ether anaesthesia. Cardiac blood was collected with a 5-ml syringe and allowed to clot in an aluminium-free polypropylene tube (13 ml, 9.5 × 1.6 cm, Sarstedt No. 55.518, Nümbrecht, W-Germany). After centrifugation at 4000 *g* for 15 min, serum was separated and stored at −20 °C to be analysed later.

Gel filtration chromatography

Gel filtration was performed on a Pharmacia C-series column, 1.6 × 100 cm (Pharmacia Fine Chemicals No. 19-5104-01, Uppsala, Sweden). Sephacryl S-200 SuperFine (Pharmacia Fine Chemicals No. 17-0871-01, Uppsala, Sweden) was provided as a ready-to-use slurry. The column was packed with the slurry according to the manufacturer's manual.

The column eluent contained per litre: 140 mmol sodium (130 mmol NaCl, 6.8 mmol NaOH, and 3.0 mmol NaN_3), 1.1 mmol $CaCl_2$, 0.50 mmol $MgCl_2$, 4.0 mmol KCl, and 10 mmol tris-(hydroxymethyl)-aminomethane (2-amino-2-hydroxymethyl-1,3-propanediol, Fluka AG No. 93352, Buchs SG Switzerland) adjusted to pH 7.40 ± 0.05 at 4 °C with 1 mol/l HCl. Both column and reservoir for the eluent were placed in a climate chamber (4 °C). The flow rate was maintained with a Perkin Elmer series 100 Pump (Perkin Elmer, Norwalk Conn., USA). The eluent fractions were collected into aluminium-free polypropylene tubes using an automated fraction collector (LKB-Producter AB 2211 SuperRac, Bromma, Sweden).

Calibration of the column was performed with a Pharmacia calibration kit (Pharmacia Fine Chemicals No. 17-0442-01, Uppsala, Sweden). Using blue dextran 2000, the void volume (Vo) was found to be 56 ml. Using ribonuclease A (relative molecular mass = 13 700), chymotrypsinogen (M_r = 25 000), ovalbumin (M_r = 43 000), albumin (M_r = 67 000), and aldolase (M_r = 158 000) a calibration curve was determined and calculated:

$$Kav = -0.44 \log (M_r) + 2.4 \quad (r = 0.97).$$

One ml of serum was applied to the gel bed, and elution was started at a flow rate of 0.41 ml/min. After elution of the void volume, collection of the fractions was started. Seventy fractions of 1.4 ml each were collected per run in polypropylene tubes and stored at 4 °C to be analysed later.

Atomic absorption spectrophotometry

Aluminium in eluent and serum was analysed by electrothermal atomic absorption spectrophotometry (Perkin Elmer 2380 and HGA 500 graphite furnace, Perkin Elmer, Norwalk, Conn., USA). Pyrolytically coated graphite tubes (Perkin Elmer, No. 091504) were used. An aluminium hollow cathode lamp (Perkin Elmer) was operated at 25 mA. Atomic absorption was measured at 309.3 nm, with a spectral band width of 0.7 nm.

The analysis of aluminium in serum has been described previously (16). For the determination of aluminium in the eluent the procedure was modified. Standard solutions containing 0, 20, 50, 100, 150, and 200 µg/l aluminium were prepared in 2 g/l Triton-X100 with 83.3 ml/l eluent. Samples were diluted 1 : 12 in 2 g/l Triton-X100. A Perkin Elmer AS-40 model autosampler was used to inject 20 µl volumes of standard solutions and samples. All aluminium analyses, both in serum and eluent, were performed in duplicate according to a previously described furnace program (16). The within-day precision of the aluminium assay in the eluent was established at 5.8% (100 µg/l; n = 10), the day-to-day precision at 6.8% (100 µg/l; n = 6), and the detection limit at 2 µg/l.

Analysis of total protein, transferrin, and citrate

Total protein was determined spectrophotometrically (Perkin Elmer 552 Norwalk, Conn., USA) with a standard Bio-Rad Microprotein Assay (17). Standards containing 0, 1, 2, 5, and 10 mg/l bovine serum albumin were used.

Transferrin in the eluent was analysed by *Laurell* "rocket" immunoelectrophoresis (18), using a LKB 2117 Multiphor (LKB-Producter AB, Bromma, Sweden). The agarose (LKB-Producter AB No. 2206-101, Bromma, Sweden), containing 1.5% rabbit anti-transferrin serum, was prepared according to the manufacturer's manual. The electrophoresis was run with 20 volts per cm and at 10 °C for 6 h. A series of 5 standards (0.05, 0.1, 0.2, 0.4, and 0.5 g transferrin per l) was applied to each plate. After electrophoresis the gel was placed in 0.2 mol/l NaCl until the following day, when it was stained with amido black and dried.

Citrate was determined spectrophotometrically (Perkin Elmer 552, Perkin Elmer, Norwalk, Conn., USA) by a standard enzymatic technique (19). Standards containing 0, 20, 40, 50, 60, 80, and 100 µmol/l Na-citrate were used.

Statistical procedures

Pearson's correlation coefficients were calculated for the relation between the serum aluminium concentration and the aluminium/transferrin molar ratio, the serum aluminium concentration and the aluminium/citrate molar ratio, and the aluminium/transferrin molar ratio and the aluminium/citrate molar ratio. A partial correlation coefficient, correcting for serum aluminium concentration, was also calculated for the aluminium/transferrin molar ratio and the aluminium/citrate molar ratio (20).

One-sided t-tests were used to examine the hypotheses that true correlations were zero. Results with a $p < 0.05$ were considered significant.

Results

Elution pattern

Gel filtration chromatography was performed with sera of 9 (4 non-treated and 5 aluminium-treated) rats containing low (less than 1 µmol/l), intermediate (between 1 and 4 µmol/l), and high (more than 4 µmol/l) concentrations of aluminium. The elution pattern of all sera on the Sephacryl S 200 SF-Column showed two major aluminium peaks. The first aluminium peak co-eluted with protein in fractions 12 to 29 (M_r between 112 000 and 32 000), with a maximum in fraction 20. The second major aluminium peak eluted in fractions 53 to 68 (M_r < 10 000), with a maximum in fractions 58 − 62. The presence of transferrin (M_r = 81 000) (21) was demonstrated in fractions 12 to 26, with a maximum in fractions 20 − 21. In fractions 55 to 65 the presence of citrate was observed, with a maximum in fractions 59 − 60 (fig. 1 and 2).

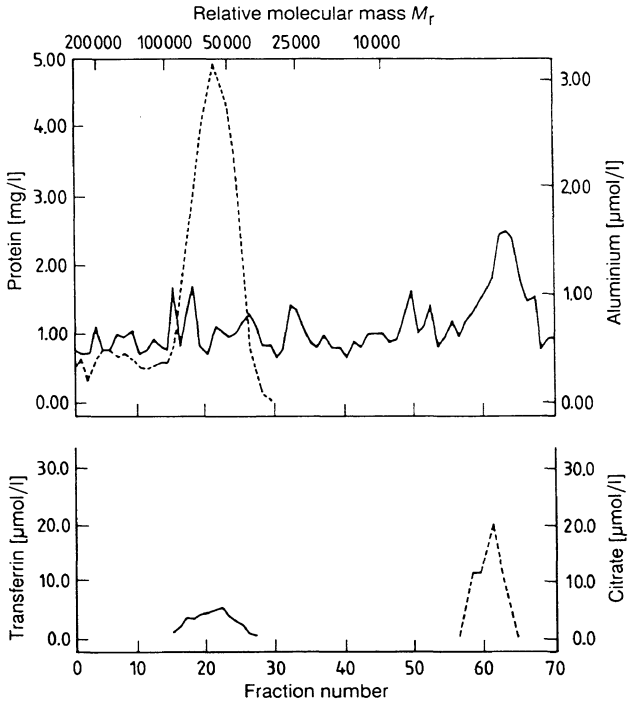

Fig. 1. Elution pattern of 1 ml rat serum containing a low concentration of aluminium (0.44 µmol/l) on a Sephacryl S-200 SF column.
The solid line in the upper part of the figure represents the aluminium concentration (in µmol/l), whereas the dotted line represents the protein concentration (in mg/l) in the elution fractions. In the lower part, however, the solid line represents the transferrin concentration (in µmol/l), whereas the dotted line represents the citrate concentration (in µmol/l) in the fractions.

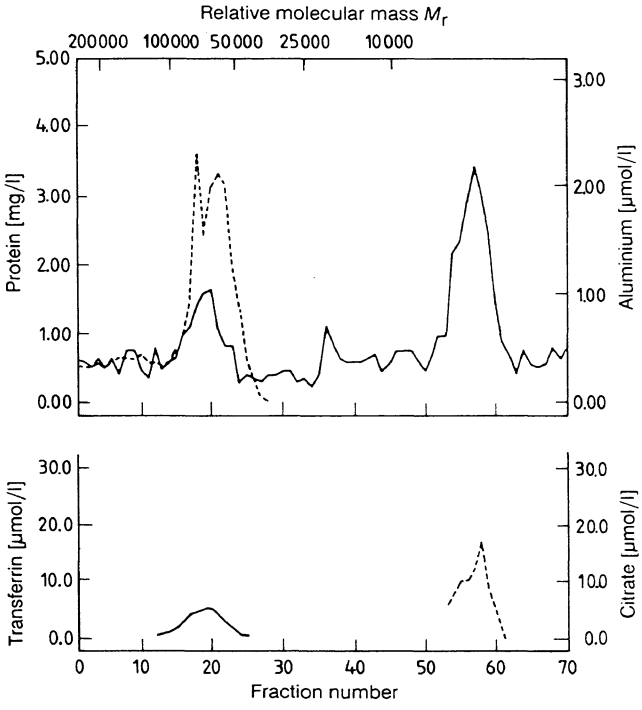

Fig. 2. Elution pattern of 1 ml rat serum containing a high concentration of aluminium (14.0 µmol/l) on a Sephacryl S-200 SF column.
The solid line in the upper part of the figure represents the aluminium concentration (in µmol/l), whereas the dotted line represents the protein concentration (in mg/l) in the elution fractions. In the lower part, however, the solid line represents the transferrin concentration (in µmol/l), whereas the dotted line represents the citrate concentration (in µmol/l) in the fractions.

Transferrin and citrate associated aluminium

For those fractions where a simultaneous elution of aluminium and transferrin was detected, the molar ratio of aluminium to transferrin was calculated. An aluminium to citrate molar ratio was determined for those fractions where a co-elution of aluminium and citrate was observed (tab. 1).

Tab. 1. Relation between serum aluminium concentration and the aluminium/transferrin and aluminium/citrate molar ratio.

Aluminium concentration in serum (µmol/l)	Aluminium/transferrin molar ratio	Aluminium/citrate molar ratio
0.26	0.339	0.120
0.44	0.326	0.081
0.44	0.189	0.100
0.82	0.347	0.110
1.78	0.350	0.140
2.67	0.334	0.141
9.41	0.079	0.102
9.48	0.223	0.096
14.0	0.260	0.151

A correlation coefficient of $r = -0.54$ ($p = 0.03$) was obtained for the relation between the serum aluminium concentration and the aluminium/transferrin molar ratio; the correlation coefficient for the serum aluminium concentration and the aluminium/citrate molar ratio was $r = 0.27$ ($p = 0.12$).

A correlation coefficient of 0.33 ($p = 0.10$) was computed for the relation between the aluminium/transferrin molar ratio and the aluminium/citrate molar ratio. However, the partial correlation coefficient between aluminium/transferrin and the aluminium/citrate molar ratio was $r = 0.59$ ($p = 0.03$).

Discussion

Gel filtration chromatography with rat sera was performed under equilibrium conditions with respect to temperature, osmolarity, and pH. The sera, containing different concentrations of aluminium (ranging from 0.44 to 14.0 µmol/l), were collected after the rats had been loaded intraperitoneally with aluminium. The elution profiles of sera showed two major alu-

minimum peaks: one co-eluted with protein (M_r between 112 000 and 32 000), while the other co-eluted with low-molecular weight serum constituents (M_r < 10 000). The separating capacity of the column was restricted to compounds with a relative molecular mass between 200 000 and 10 000. High-molecular mass proteins, such as macroglobulin, immunoglobulin M, and orosomucoid were eluted in the void volume. As these proteins were not separated, their respective association with aluminium could not be studied in the present investigation.

The presence of transferrin was demonstrated in the protein peak co-eluting with aluminium. This is consistent with findings of others (10–12), and supports the view that transferrin may act as a carrier for aluminium in plasma (22, 23). The negative correlation coefficient for the relation between the serum aluminium concentration and the aluminium/transferrin molar ratio ($r = -0.54$; $p = 0.03$) suggests that the relative amount of aluminium bound to transferrin decreases when the serum aluminium concentration increases. Increased co-elution of free aluminium in the transferrin fractions from sera with a high aluminium concentration may explain this contradictory finding. The resolution of the column used in this study, however, was insufficiently high to exclude a possible aluminium-binding role for other proteins, especially albumin (M_r = 67 000).

The second aluminium peak co-eluted with low-molecular weight serum constituents (M_r < 10 000). Although ultrafiltration studies indicated that part of aluminium in serum is non-protein bound and ultrafiltrable (12, 14, 24), co-elution of aluminium with low-molecular weight serum constituents has only been detected in some gel filtration studies (8, 9, 13), while in other studies, using other types of gel, no association of aluminium with low-molecular weight species was found (10–12).

Although citrate has often been nominated on theoretical or indirect grounds as a low-molecular weight binder for aluminium in plasma, association of aluminium with citrate has never been demonstrated before. The present finding strongly supports the postulated action of citrate as a chelator for aluminium in plasma (23, 25–27).

In addition, based upon recent reports on the effect of citrate on the intestinal absorption of aluminium (28), and on the accumulation of aluminium in bone and brain (29, 30), it is most likely that the passage of aluminium through the cell membrane is markedly increased when complexed to citrate. The aluminium/citrate complex might therefore be of paramount importance for the intracellular accumulation of aluminium.

The ultrafiltrable aluminium fraction is reported to increase with the total serum aluminium concentration (9, 14). For the aluminium/citrate molar ratio, however, this could not be confirmed in the present study. This ratio appeared not to depend on the serum aluminium concentration ($r = 0.37$; $p = 0.10$).

Application of partial correlation calculations revealed that the aluminium/transferrin and the aluminium/citrate molar ratios are significantly correlated ($p = 0.03$). This observation suggests an association between transferrin and citrate in the binding of aluminium ions in plasma; and it supports the view that the behaviour of aluminium in plasma is very similar to that of iron (III), in which citrate acts as a chelator and the iron/citrate complex reacts with transferrin to form the transport complex (31).

Regarding the fraction of serum aluminium which co-eluted with both transferrin and citrate, it was calculated that, on average, only 21% (SD = 2.5%; n = 9) of the total amount of aluminium recovered in 70 fractions was found in the transferrin peak, and that only 23% (SD = 8.2%; n = 9) of the aluminium co-eluted with citrate. The recovery in the 70 fractions, however, was greater than the quantity of aluminium in the serum applied to the gel column. Contamination of the eluent with aluminium may be responsible for this. The aluminium/transferrin molar ratio was generally larger than the aluminium/citrate molar ratio (tab. 1), confirming that the affinity of aluminium for transferrin is greater than for citrate (23).

In conclusion, the observations of others that transferrin plays a role in the binding and transport of aluminium in serum was confirmed in this study. A comparable role for other proteins like albumin could, however, not be excluded.

In addition to transferrin, aluminium was shown to be associated with citrate in serum, resulting in a low-molecular weight complex. In view of other studies in which citrate was shown to enhance aluminium transport in tissue, it may be assumed that the aluminium/citrate complex in serum may play an important role in the intracellular accumulation, and hence toxicity, of aluminium.

Acknowledgement

The authors wish to thank Dr. *A. H. Zwinderman* (Department of Medical Statistics) for statistical advice.

References

1. Flendrig, J. A., Kruis, H. & Das, H. A. (1976) Aluminium and dialysis dementia. Lancet *II*, 1235.
2. Alfrey, A. C., LeGendre, G. R. & Kaehny, W. D. (1976) The dialysis encephalopathy syndrome. Possible aluminium intoxication. N. Engl. J. Med. *294*, 184–188.
3. Wills, M. R. & Savory, J. (1983) Aluminium poisoning: Dialysis encephalopathy, osteomalacia, and anaemia. Lancet *II*, 29–34.
4. Crapper, D. R. Quittkat, S., Krishanan, S. S., Dalton, A. J. & De Boni, U. (1980) Intranuclear aluminium content in Alzheimer's disease, dialysis encephalopathy, and experimental aluminium encephalopathy. Acta Neuropathol. *54*, 19–24.
5. Yoshimashu, F., Uebayashi, Y., Yase, Y., Iwata, S. & Sasajima, K. (1976) Studies on amyotrophic lateral sclerosis by neutron activation analysis. Folia Psychiatr. Neurol. Japon. *30*, 49–55.
6. Yoshimashu, F., Yasui, M., Yase, Y., Iwata, S., Gajdusek, D. G., Gibbs, C. J. & Chen, K. M. (1980) Studies on amyotrophic lateral sclerosis by neutron analysis. 2. Comparative study of analytical results on Guam PD, Japanese ALS and Alzheimer disease cases. Folia Psychiatr. Neurol. Japon. *34*, 75–82.
7. Ganrot, P. O. (1986) Metabolism & possible health effects of aluminium. Environ. Health Perspect. *65*, 363–441.
8. King, S. W., Wills, M. R. & Savory, J. (1979) Serum binding of aluminium. Res. Comm. Chem. Pathol. Pharmacol. *26*, 161–166.
9. King, S. W., Savory, J. & Wills, M. R. (1982) Aluminum distribution in serum following hemodialysis. Ann. Clin. Lab. Sci. *12*, 143–149.
10. Trapp, G. A. (1983) Plasma aluminum is bound to transferrin. Life Sci. *33*, 313–316.
11. Cochran, M. C., Patterson, D., Neoh, S., Stevens, B. & Mazzachi, R. (1985) Binding of Al in plasma of patients on maintenance hemodialysis. Clin. Chem. *31*, 1314–1316.
12. Rahman, H., Skillen, A. W., Channon, S. M., Ward, M. K. & Kerr, D. N. S. (1985) Methods for studying the binding of aluminum by serum protein. Clin. Chem. *31*, 1969–1973.
13. Bertholf, R. L., Wills, M. R. & Savory, J. (1984) Quantitative study of aluminum binding to human serum albumin and transferrin by a chelex competitive binding assay. Biochem. Biophys. Res. Comm. *125*, 1020–1024.
14. Elliott, H. L., MacDougall, A. I., Fell, G. S. & Gardiner, P. H. E. (1978) Plasmaphereses, aluminium and dialysis dementia. Lancet *II*, 1255.
15. Toffaletti, J., Savory, J. & Gitelman, J. J. (1977) Use of gelfiltration to examine the distribution of calcium among serum proteins. Clin. Chem. *23*, 2306–2310.
16. Van der Voet, G. B., De Haas, E. J. M. & De Wolff, F. A. (1985) Monitoring of aluminium in whole blood, plasma, serum, and water by a single procedure using flameless atomic absorption spectrophotometry. J. Anal. Tox. *9*, 97–100.
17. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*, 248–254.
18. Laurell, C. B. (1966) Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal. Biochem. *15*, 45–52.
19. Tompkins, D. & Toffaletti, J. (1982) Enzymatic determination of citrate in serum and urine, with use of the Worthington "Ultrafree" device. Clin. Chem. *28*, 192–195.
20. Lindeman, R. H., Merenda, P. S. & Good, R. Z. (1981) *Introduction to bivariate and multivariate analysis*, pp. 11–117, Scott, Foresman, & Company, London.
21. Van Eijk, H. G. & Van Noort, W. L. (1976) Isolation of rat transferrin using CNBr-activated Sepharose 4B. J. Clin. Chem. Clin. Biochem. *14*, 475–478.
22. Trapp, G. A. (1986) Interactions of aluminum with cofactors, enzymes, and other proteins. Kidney Int. *29*, S12–S16.
23. Martin, B. R. (1986) The chemistry of aluminum as related to biology and medicine. Clin. Chem. *32*, 1797–1806.
24. Lundin, A. P., Caruso, C., Sass, M. & Berlyne, G. M. (1978) Ultrafiltrable aluminum in serum of normal man. Clin. Res. *26*, 636A.
25. Jackson, G. E. (1983) Studies on the chelation of aluminium for biological application. Part I. Citric acid. S. Afr. J. Chem. *35*, Part III, 89–96.
26. Öhman, L. O. & Sjöberg, S. (1983) Equilibrium and structural studies of silicone (IV) and aluminium (III) in aqueous solution. A potentiometric study of mono- and polynuclear aluminium (III) citrate. J. Chem. Soc. Dalton Trans. *3*, 2513–2517.
27. Birchall, L. D. & Chappell, J. S. (1988) The chemistry of aluminum and silicon in relation to Alzheimer's disease. Clin. Chem. *34*, 265–267.
28. Van der Voet, G. B., Van Ginkel, M. F. & De Wolff, F. A. (1989) Intestinal absorption of aluminum in rat: Energy-dependent and -independent processes. Tox. Appl. Pharm. *99*, 90–97.
29. Slanina, P., Falkeborn, Y., Frech, W. & Cedergren, A. (1984) Aluminium concentrations in the brain & bone of rats fed citric acid, aluminium citrate or aluminium hydroxide. Fed. Chem. Toxic. *22*, 391–397.
30. Slanina, P., Frech, W., Bernhardson, A., Cedergren, A. & Mattson, B. (1985) Influence of dietary factors on aluminium absorption and retention in the brain and bone of rat. Acta Pharmacol. Toxicol. *56*, 331–336.
31. Bates, G. W., Billups, C. & Saltman, P. (1967) The kinetics and mechanism of iron (III) exchange between chelates and transferrin. I. The complexes of citrate and nitriloacetic acid. J. Biol. Chem. *242*, 2810–2815.

Prof. Dr. F. A. de Wolff
Toxicology Laboratory
P. O. Box 9600
NL-2300 RC Leiden